

System-L amino acid transporters play a key role in pancreatic β -cell signalling and function.

Qi Cheng^{2,4}, Violeta D Beltran^{2,3}, Stanley MH Chan¹, Jeremy R Brown³, Alan Bevington³
and Terence P Herbert^{1,2}

¹Health Innovations Research Institute (HIRI), School of Medical Sciences, RMIT University, PO Box 71, BUNDOORA, VIC 3083, Australia;

² Previously at the Department of Cell Physiology and Pharmacology, University of Leicester, LE1 5HN, UK;

³Department of Infection, Immunity and Inflammation, University of Leicester, LE1 5HN, UK;

⁴Research School of Biology, Australian National University, Acton, ACT 2601, Australia.

Abbreviated title: System-L transporters and the pancreatic β -cell

Key terms: Diabetes, islets, cellular signalling, amino acid transporters, mTORC1

Word count: (excluding abstract, figure captions, and references) 5671

Number of figures and tables: 7 figures and 0 tables

Corresponding author and person to whom reprint requests should be addressed

Associate Professor Terence P. Herbert

Health Innovations Research Institute (HIRI)

School of Medical Sciences

RMIT University

PO Box 71

28 BUNDOORA VIC 3083

29 Australia

30 Tel: +61 3 9925 7339

31 Fax: +61 3 9925 7466

32 Email: terence.herbert@rmit.edu.au

33

34 Grants or fellowships supporting the writing of the paper

35 VDB was supported by a Scholarship from the Programa Doctoral Becas COLCIENCIAS

36 Administrado por Colfuturo Bogotá, Colombia

37

38 **Disclosure statement:** The authors have nothing to declare.

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Abstract

The branched-chain amino acids (BCAA) leucine, isoleucine and valine, are essential amino acids that play a critical role in cellular signalling and metabolism. They acutely stimulate insulin secretion and activate the regulatory serine/threonine kinase mammalian target of rapamycin complex 1 (mTORC1), a kinase that promotes increased β -cell mass and function. The effects of BCAA on cellular function are dependent on their active transport into mammalian cells via amino acid transporters and thus the expression and activity of these transporters likely influences β -cell signalling and function. In this report we show that the System-L transporters are required for BCAA uptake into clonal β -cell lines and pancreatic islets and that these are essential for signalling to mTORC1. Further investigation revealed that the System-L transporter LAT1 is abundantly expressed in islets and that knock-down of LAT1 using siRNA inhibits mTORC1 signalling, leucine-stimulated insulin secretion and islet cell proliferation. In summary, we show that the System-L transporter LAT1 is required for regulating β -cell signaling and function in islets and thus may be a novel pharmacological/nutritional target for the treatment and prevention of type-2 diabetes.

Introduction.

The branched-chain amino acids (BCAA), leucine, isoleucine, and valine, are essential amino acids that play an important role in glucose homeostasis. *In vitro*, leucine stimulates insulin secretion (Newsholme *et al.* 2007) and improves insulin secretory function of human islets (Yang *et al.* 2006). Studies on the effect of diets containing increased BCAA or leucine on glucose homeostasis *in vivo* have been contradictory, likely due to differences in diet regimens and models. However, there is evidence that high protein diets, a source of BCAA, or leucine supplementation enhances insulin secretion and glucose clearance in healthy adults and type-2 diabetics (Linn *et al.* 1996; van Loon *et al.* 2003; Kalogeropoulou *et al.* 2008; Leenders & van Loon 2011). Moreover, human neonates fed high protein formula milk have significantly raised serum leucine which parallels increased C-peptide (Socha *et al.* 2011).

Branch chain amino acids, and particularly leucine, are also critical for the activation of mammalian target of rapamycin complex 1 (mTORC1) as they promote the translocation of mTORC1 to GTP-

loaded Rheb, its upstream activator located at the lysosome (for review see (Bar-Peled & Sabatini 2014)). This process is dependent on a complex of proteins including Rags (Ras-related GTPases), the Ragulator, v-ATPase, GATOR (GAP activity towards Rags), and folliculin. Yet how changes in the amino acid concentration are sensed is still unclear. Although there is growing evidence to suggest that changes in the intra-lysosomal concentration play a significant role. Importantly, mTORC1 is a kinase whose hyperactivation in mice has been shown to stimulate both pancreatic β -cell function and mass (for reviews see (Blandino-Rosano *et al.* 2012; Xie & Herbert 2012)). For example, β -cell specific deletion of tuberous sclerosis complex-2 (Tsc2), a negative regulator of mTORC1, or β -cell specific expression of constitutively-active Rheb, a small G protein which binds to and activates mTORC1, results in increased β -cell mass due to increased proliferation and cell size (Rachdi *et al.* 2008; Balcazar *et al.* 2009). Mice expressing constitutively-active PKB in β -cells also have increased β -cell mass due to increased mTORC1 activity (Elghazi *et al.* 2007). Therefore, BCAA dependent activation of mTORC1 may be important in β -cell compensation and hence in protecting against the development of type-2 diabetes.

The effects of amino acids on β -cell signaling, function and mass are likely mediated through changes in their intracellular concentration. Importantly, amino acid transporters play an important part in determining the size and composition of the intracellular amino acids pool. There are several amino acid transporter Systems facilitating transport of BCAAs including System B^{0,+}, System y⁺L and System b^{0,+**} and System-L (Hyde *et al.* 2003). The System-L amino acid transporters (LATs) are high-affinity transporters of large neutral amino acids (i.e. the BCAA and aromatic amino acids) that are competitively inhibited by 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), a non-metabolisable analogue of L-leucine. There are four members of this group called large neutral amino acid transporters or LATs. LAT1 and 2 are heterodimeric and are composed of a 'light subunit' that serves as an amino acid permease and a 'heavy subunit': glycoprotein CD98 (cluster of differentiation 98) protein (encoded by SLC3A2) aka 4F2hc (4F2 cell-surface antigen heavy chain), that facilitates translocation of LAT to the plasma membrane (Verrey 2003). In contrast LAT3 and LAT4 are made up of a single subunit and doesn't require CD98 for transport activity (Bodoy *et al.*

2005). Surprisingly, the amino acid transporter/s responsible for the uptake of BCAA into β -cells has remained largely unexplored. Moreover, their potential role in regulating mTORC1 activity and β -cell function are essentially unknown. In this report we demonstrate that System-L transporters are required for signalling to mTORC1 in β -cells. We also show that LAT1 is the most predominately expressed transporter in islets and that this transporter plays an important role in regulating both β -cell signalling and function.

Materials and Methods

Chemicals and media. All chemicals and media were purchased from Sigma-Aldrich (St. Louis, MO, USA) otherwise stated. EBSS (Earle's Balanced Salt Solution) contained 20mM glucose, 2mM L-glutamine, P/S/N (50 μ g/ml streptomycin, 50 units/ml penicillin sulphate and 0.1mg/ml Neomycin) and the pH adjusted to 7.4 using NaHCO_3 . 1x MEM essential amino acids solution was made from a 50X MEM stock (Sigma M5550) and contained 0.1264g/l L-Arg•HCl; 0.03128g/l L-Cys•2HCl; 0.042g/l L-His HCl•H₂O; 0.0525g/l L-Ile; 0.0524g/l L-Leu; 0.0725g/l L-Lys•HCl; 0.0151g/l L-Met; 0.033g/l L-Phe; 0.0476g/l L-Thr; 0.0102g/l L-Typ; 0.036g/l L-Tyr; 0.0468g/l L-Val. 1x MEM essential amino acid mixture without System-L amino acids was made using individual amino acids purchased from Sigma and contained 0.1264g/l L-Arg•HCl; 0.03128g/l L-Cys•2HCl; 0.042g/l L-His HCl•H₂O; 0.0725g/l L-Lys•HCl; 0.0151g/l L-Met; 0.0476g/l L-Thr. DMEM minus leucine media was made in-house from its constituent components.

Cell culture and treatment. Rat insulinoma 1E (INS1E) cells (Merglen *et al.* 2004) were maintained in a humidified incubator (95% air, 5% CO_2) at 37°C in RPMI 1640 medium containing 11.1mM glucose, supplemented with 5% (v/v) heat inactivated fetal bovine serum (FBS) (Invitrogen, Thermo Scientific Inc, Waltham, MA USA), 1mM sodium pyruvate, 10mM HEPES, 55 μ M β -mercaptoethanol, P/S/N (50 μ g/ml streptomycin, 50 units/ml penicillin sulphate and 0.1mg/ml Neomycin). Prior to treatments, INS1E cells were serum starved overnight (16h) in CMRL-1066 medium containing 0.5mM L-glutamine and P/S/N. Cells were treated as described in the figure legends.

Pancreatic islets were isolated from Male Wistar Albino rats weighing 200g-250g and dissociated as previously described (Moore *et al.* 2011). The dispersed cells were plated on a poly-D-lysine coated plates and incubated in RPMI-1640 media containing 10% FBS and P/S/N. Cells were treated as described in the figure legends.

SDS- Polyacrylamide Gel Electrophoresis and Western Blot Analysis.

SDS-page and Western blotting were performed as previously described (Moore *et al.* 2009) using anti-phospho Akt/PKB (Ser473), anti-Akt/PKB, anti-phospho S6K1(Thr389), anti-phospho ribosomal protein S6 (Ser240 /244) and anti-LC3 A/B from Cell signalling Technologies, Beverly, MA, USA, and anti-ribosomal protein S6 obtained from Santa Cruz Biotechnology Inc, Santa Cruz, CA USA.

Amino Acid Analysis.

Amino Acid analysis was performed essentially as previously described (Evans *et al.* 2007). After treatments, INS1E or islets of Langerhans were rapidly chilled on ice, rinsed three times with ice-cold 1x PBS to remove extracellular amino acids, and deproteinized by scraping with 150µl of 0.3M perchloric acid. The resulting lysate was transferred to microcentrifuge tubes on ice, followed by incubation for at least 30 min. Precipitated protein was sedimented by centrifugation (10min, 4°C, 14000g) and retained for total protein assay. Supernatant was filtered through a 0.45µm microfilter and was immediately used for determination of amino acids on an Agilent 1100 high-performance liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) with Zorbax Eclipse AAA column (4.6 x 75mm, 3.5µm) at 40°C with *o*-phthalaldehyde/3-mercaptopropionate/9-fluorenylmethylchloroformate precolumn derivatization and ultraviolet and fluorimetric postcolumn detection.

RNA isolation of qRT-PCR

peqGOLD Total RNA Kit (VWR international, Germany) was used in RNA extraction for qRT-PCR according to manufacturer's protocol. Islets of Langerhans were cultured for 2 days prior to RNA extraction. 2µg of total RNA from each extraction was used as template for reverse-transcription

using SuperScriptTM VILO cDNA synthesis kit (Invitrogen). qRT-PCR was then performed on cDNA from 1ng RNA using gene-specific primers from PrimerDesign (Southampton, UK): Rat LAT1 forward 5'-TTGTTTCGTTTCAGTAGCACATTG-3' and reverse 5'-ATTCATCGTCCGTTTGTTCAGT-3'; Rat LAT2 forward 5'-CCAGTTCCTCTCCCCTCCT-3' and reverse 5'-CAAAGTGAGTGCCATCCTGTC-3'; Rat LAT3 forward 5'-ACACTGAAGTTTTGTCTCCGTTG-3' and reverse 5'-TTGGGCAGAGTAAGTGAGGTAAA-3'; Rat LAT4 forward 5'-CAGAAGCGAGACAGGCAGAT-3' and reverse 5'-TGTAGAGGCAGATTAGGAATGAGG-3'; and 18s RNA primers forward 5'-GTTGGTTTTTCGGAAGTGGG-3' and reverse 5'-GCATCGTTTATGGTCGGAAC-3'.

Transfection of siRNAs

Rat islets of Langerhans were dispersed in a 24-well plate and cultured overnight in rat islet growing medium prior to transfection using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM® medium (Invitrogen) according to the manufacturer's instructions. For LAT1 knock-down, the cells were transfected for 72h with 100nM on-target plus siRNA (Dharmacon) against SLC7A5 (L-092749-01-0005), or 100nM Silencer® Select Pre-Designed siRNA (Invitrogen) against SLC7A5 (s132356).

Proliferation Assay

Rat islet cell proliferation assays were performed essentially as previously described (Kwon *et al.* 2006; Xie *et al.* 2014). 150 rat islets of Langerhans per treatment were used for proliferation assay. Islet cells were starved in RPMI media supplemented with 0.2% FBS and treated with DMEM+16.7mM glucose in the absence or presence of 0.8mM L-Leucine or 2.5mM BCH for 48 h. 2μCi/ml [³H]-thymidine was added during the last 24h. Cells were then collected and washed twice with 1X Phosphate Buffered Saline (PBS). DNA was precipitated on ice upon the addition of 5% trichloroacetic acid for 30min. Samples were spun at 16000 x g, 4°C for 10min and DNA pellets were then solubilised by adding 0.1N NaOH and incubated for 30min at RT. [³H]-thymidine incorporation was determined by quench-corrected scintillation counting using the LS6500 multi-purpose scintillation counter to detect DPM (disintegrations per minute) in each sample.

Leucine transport assays.

Cells were preincubated for 10 min in the culture incubator in Leucine-free Krebs Ringer Buffer (KRB). L-[4,5-³H]-leucine (Perkin Elmer) plus unlabelled L-Leucine were then added to the KRB to give a final radio-isotope concentration of 5μCi/ml and a final total L-Leucine concentration of 0.8mM. BCH was also added to some cultures to give a final concentration of 2.5mM. Cultures with ³H were then incubated in the culture incubator for exactly 5 min. The cells were then immediately placed on ice, the medium aspirated off, and the cells rapidly washed 3x in ice-cold KRB. 0.05M NaOH was then added to the cells and the lysates transferred to microcentrifuge tubes. The lysates were then incubated at 70°C for 30 min. A fraction of the lysate was then transferred to a scintillation vial containing Ecoscint A scintillant and allowed to stand for at least an hour to allow chemiluminescence to decay before quench-corrected scintillation counting using the LS6500 multi-purpose scintillation counter. L-[4,5-³H]-leucine transport rate is expressed as DPM / mg protein / 5 min.

Insulin Secretion Assay.

Following treatments, the test media were collected and centrifuged at 2000g for 3 min to pellet any detached cells. The supernatants were collected and the insulin concentrations measured using a Rat Insulin Enzyme Immunoassay Kit (Bertin Pharma, A05105-96 wells) with rat insulin as a standard in accordance with manufacturer's instructions. The absorbance was read at 414nm on the NOVostar microplate reader.

Statistical Analysis.

Results are expressed as means ± SEM. Data were analyzed by one-way analysis of variance, ANOVA, followed by Bonferroni correction for all pair-wise comparisons, or two-tailed paired T-test using GraphPad Prism 6.0. Significance was assigned at P< 0.05.

Results and Discussion

System-L transporters are critical for mTORC1 activation in the rodent clonal pancreatic β -cell line INS1E.

The System-L amino acid transporters are widely expressed and members of its family are capable of transporting large neutral amino acids including the BCAA. Therefore, we initially investigated the role of the System-L family of amino acid transporters in signalling to mTORC1 in β -cells.

Clonal rat pancreatic β -cells (INS1E cells) were treated with insulin to activate mTORC1, in the presence or absence of BCH (2-aminobicyclo-(2,2,1) heptanecarboxylic acid), a competitive inhibitor of System-L, or in the presence or absence of the System-L amino acids substrates: L-Leu, L-Ile, L-Val, L-Phe, L-Tyr and L-Trp (Figure 1a). As anticipated, the addition of insulin led to a robust increase in mTORC1 activity as determined by the phosphorylation of its downstream target S6 kinase 1 (S6K1) on Thr389 and a substrate of S6K1, ribosomal protein S6 (rpS6) on Ser240/244. Insulin also stimulated the activation of protein kinase B (PKB), as assessed by its phosphorylation on Ser473 (Figure 1a). The addition of BCH or the withdrawal of System-L substrates (i.e. L-Leu, L-Ile, L-Val, L-Phe, L-Tyr and L-Trp) significantly inhibited insulin-stimulated mTORC1 activation. In addition, BCH induced the conversion of cytosolic LC3-I to the lipid-bound LC3-II, which is widely used as the measurement of autophagic flux (Klionsky *et al.* 2008), indicating that BCH promotes autophagy, possibly through its inhibition of mTORC1 (Jewell *et al.* 2013). Interestingly, BCH also inhibited insulin-stimulated phosphorylation of PKB. In contrast, the withdrawal of System-L amino acids had no significant effect on the conversion of LC3 or on the phosphorylation of PKB. In summary, these results provide good evidence that the System-L transporters play a critical role in signalling to mTORC1 and may also influence autophagy and PKB activity.

The effects of BCH and the removal of System-L substrates on cellular signalling are possibly mediated by a decrease in the intracellular concentration of BCAA. To investigate this, the effect of BCH or System-L AA withdrawal on the intracellular amino acids pool was determined by HPLC analysis (Figure 1b). System-L AA withdrawal led to a dramatic decrease in the intracellular concentration of the BCAA but had no significant effect on the intracellular concentration of the

aromatic amino acids, likely reflecting their low rate of catabolism. The addition of BCH caused a significant decrease in the intracellular concentration of leucine and a trend towards a decrease in the other BCAA, indicating the importance of System-L transporters in the uptake of BCAA in β -cells. To directly investigate the role of System-L transporters on BCAA transport into INS1E cells, the effect of BCH on [3 H]-leucine uptake into INS1E cells was also determined (Figure 1c1e). BCH caused a significant decrease in the uptake of [3 H]-leucine, demonstrating that System-L transporters play an important role in BCAA uptake into INS1E cells.

The uptake of L-leucine through System-L amino transporters is required for mTORC1 activation.

To determine which System-L amino acids are required for signalling to mTORC1, INS1E cells were depleted of System-L amino acids and the effect of the re-addition of each System-L amino acid in turn on the restoration of insulin signalling to mTORC1 was assessed (Figure 2a). The re-addition of L-Leucine significantly restored insulin signalling to mTORC1, whereas the re-addition of L-Isoleucine, L-Valine, L-Phenylalanine, L-Tyrosine and L-Tryptophan had little-to-no effect on mTORC1. Thus, of the System-L substrates, only L-Leucine is able to restore insulin signalling to mTORC1. These results confirm and extend previous findings in the rat insulinoma cell line RINm5F and isolated rat islets (Xu *et al.* 1998) .

To investigate whether L-Leucine-stimulated mTORC1 activation was indeed dependent upon System-L transporters, INS1E cells depleted of leucine were incubated with 0.8mM or 0.4mM L-Leucine in the presence or absence of 10mM BCH (Figure 2b). As anticipated, the re-addition of leucine-stimulated mTORC1 activation as determined by the phosphorylation of S6K1 and rpS6. Surprisingly, leucine re-addition also inhibited the phosphorylation of PKB on S473 (Figure 2b). This is possibly mediated by a well characterised mTORC1-dependent feed-back mechanism in which S6K phosphorylates insulin receptor substrate and inhibits insulin signalling (Harrington *et al.* 2004; Tremblay *et al.* 2007). Importantly, in the presence of BCH, leucine-stimulated mTORC1 activation was inhibited. Moreover, BCH inhibited PKB and induced autophagy as assessed by the conversion of cytosolic LC3-I to the lipid-bound LC3-II (Figure 2b).

In order to define the EC₅₀ of L-Leucine to activate mTORC1, INS1E cells preincubated in leucine-free media, were treated for 30 min with increasing concentrations of L-Leucine (Figure 2c). L-Leucine activated mTORC1 in a dose-dependent manner with EC₅₀'s of 267 μ M and 227 μ M for S6K1 and rpS6 phosphorylation respectively, with a maximum response at approximately 2mM (Figure 2c). Nifedipine, an L-type VGCC inhibitor, is unable to block leucine-stimulated activation of mTORC1 (figure 2d) and thus the effects of leucine on mTORC1 are not due to leucine-stimulated insulin secretion.

To investigate the temporal regulation of mTORC1 activation upon leucine re-addition or withdrawal, INS1E cells were either depleted of leucine (Figure 3a) or leucine added to leucine-starved cells (Figure 3b) for up to 30 min and changes in PKB and mTORC1 activity determined (Figure 3). The phosphorylation of S6K1 at Thr389 and rpS6 rapidly decreased within 10min of L-Leucine withdrawal, and further decreased to an undetectable level by 15min (Figure 3a). The phosphorylation of PKB at Ser473 was also significantly decreased by 5min of L-Leucine withdrawal; however, by 15min, PKB phosphorylation started to recover and returned to basal levels by 30min (Figure 3a). In addition, the conversion of LC3-I to LC3-II increased within 5min of L-Leucine withdrawal, indicating the induction of autophagy (Figure 3a). The re-addition of leucine to leucine-starved cells caused a rapid (within 5-10min) increase in mTORC1 activation as determined by the phosphorylation of S6K1 and rpS6 (Figure 3b). The phosphorylation of PKB at Ser473 also rapidly decreased but returned to basal levels by 30min (Figure 3b). In addition, the abundance of LC3-II rapidly decreased, indicating an inhibition of autophagy (Figure 3b). Given the rapidity of these events, we investigated whether these changes in mTORC1 activity correlated with changes in the intracellular leucine concentration (Figure 3c). The withdrawal of extracellular leucine for 10 min resulted in the depletion of intracellular leucine whereas within 10 min of its re-addition the intracellular concentration of leucine returned to basal levels (Figure 3c). Thus under these conditions changes in the extracellular concentration of leucine are rapidly mirrored by changes in their intracellular concentration, which reflect the activation state of mTORC1.

System-L transporters are required for islet cell function and signalling to mTORC1.

Having established the importance of System-L transporters in clonal pancreatic β -cells, we wished to investigate their potential role in primary β -cells (figure 4). Therefore, islets of Langerhans were isolated from rats and deprived of leucine prior to the re-addition of the system-L substrate L-Leucine in the presence or absence of BCH (Figure 4a). The re-addition of L-Leucine significantly stimulated mTORC1, as determined by the phosphorylation state of S6K1 at Thr389 and rpS6 on S240/244, and reduced the phosphorylation of PKB at Ser473. The co-addition of BCH significantly inhibited leucine-stimulated mTORC1 activation and also induced autophagy as measured by the conversion of LC3-I to LC3-II (Figure 4a). These results are similar to that observed in INS1E cells (Figure 2). Leucine can also stimulate insulin secretion (Newsholme *et al.* 2007), so in order to ascertain the role of System-L transporters in leucine-stimulated insulin secretion, rat islets of Langerhans were incubated in leucine depleted media for 1h prior to addition of leucine for 30min in the presence or absence of BCH. As anticipated, leucine stimulated insulin secretion but this was significantly inhibited by the presence of BCH (Figure 4b), showing that leucine-dependent stimulation of insulin secretion is dependent upon System-L transport activity. As mTORC1 activation promotes β -cell proliferation (Xie *et al.* 2014) the role of System-L transporters on islet cell proliferation was also investigated. Dispersed islets were incubated in either in the absence of L-leucine or in the presence of BCH and islet cell proliferation was measured by the incorporation of [3 H]-thymidine into DNA (Figure 4c). The addition of BCH or the removal of leucine significantly inhibited islet cell proliferation. Thus system-L transporter activity is required for islet cell proliferation.

To investigate the effect of BCH or leucine withdrawal on the intracellular leucine concentration HPLC amino acid analysis was performed (figure 4d). The removal of leucine caused the depletion in leucine whereas its re-addition restored intracellular leucine concentration. The re-addition of leucine in the presence of BCH caused a significant decrease in the intracellular concentration of leucine indicating that leucine uptake is mediated by System-L transporter (Figure 4d). Moreover [3 H]-leucine uptake into cells was significantly inhibited by BCH (figure 4e) providing further evidence that System-L transporters play a significant role in leucine transport into islets.

System-L transporter expression and function in islets of Langerhans.

Having established that system-L transporters play a significant role in β -cell signalling, we wished to investigate which of the System-L transporters were likely responsible. There are four System-L amino acid: LAT1, LAT2, LAT3 and LAT4 encoded by *SLC7A5*, *SLC7A8*, *SLC43A1* and *SLC43A2* respectively (Kanai *et al.* 1998; Mastroberardino *et al.* 1998; Pineda *et al.* 1999; Segawa *et al.* 1999). In order to assess their relative expression in primary rat islets, qRT-PCR (quantitative real time polymerase chain reaction) was performed (Figure 5a). LAT1, LAT2 and LAT4 were all expressed in islets, although LAT1 and LAT4 were found to be the most abundantly expressed System-L transporters.

Both LAT1 and 2 are obligate heterodimers that require CD98 for function. Therefore, to inhibit the function of both LAT1 and 2 and not LAT3 and 4 the expression of CD98 was down regulated using siRNA (figure 5b). Knockdown of CD98 caused a significant decrease in the activation of mTORC1 as determined by the phosphorylation of S6K. There was also a trend towards a decrease in both PKB and rpS6 phosphorylation but these changes proved statistically insignificant. Taken together these data would suggest that LAT1 likely plays a significant role in regulating mTORC1 in β -cells

LAT1 plays an important role in islet signalling to mTORC1 in islets of Langerhans

As LAT1 is abundantly expressed System-L transporter in islets and siRNA mediated knockdown of CD98 inhibits mTORC1 (Figure 5), we focused on the role of LAT1 in islets. Two distinct siRNAs directed against the mRNA encoding LAT1 (*slc7a5*) (labelled LAT1-A and LAT1-B) were transfected into dispersed islet cells (Figure 6). The efficiency of LAT1 knockdown, as determined by qRT-PCR quantification, was approximately 41% and 59% using siRNA's LAT1-A and LAT1-B respectively (Figure 6c and d). The knockdown of LAT1 expression using either siRNA had no detectable effect on cell morphology (unpublished observations) but caused a significant decrease in L-Leucine-stimulated phosphorylation of S6K1 at Thr389, demonstrating that LAT1 is required for mTORC1 activation (Figure 6a and b). It also consistently caused a decrease in the phosphorylation of rpS6 at Ser240/244; however this proved statistically insignificant. Interestingly the knock down of

LAT1 using siRNA LAT1-B significantly inhibited PKB phosphorylation. Although no statistically significant change in the phosphorylation of PKB was detected using siRNA-A, there was a trend towards a decrease in PKB phosphorylation.

In conclusion, the System-L transporter LAT1 plays an important role in signalling to mTORC1 and possibly PKB in islet cells.

LAT1 plays an important role in islet function and proliferation in islets of Langerhans

To investigate the role of LAT1 in islet function and proliferation the expression of LAT1 was knocked-down in dispersed islets of Langerhans using siRNA. siRNA mediated reduction in LAT1 expression significantly inhibited leucine-stimulated insulin secretion (Figure 7a). Moreover, islet cell proliferation, as determined by the incorporation of [³H]-thymidine into DNA, was also significantly inhibited (Figure 7b). In conclusion, the System-L transporter LAT1 plays an important role in islet cell proliferation and insulin secretion. Although a reduction in LAT1 expression decreases mTORC1 activation and others and we have shown that mTORC1 is required for β -cell proliferation (Balcazar *et al.* 2009; Xie *et al.* 2014) it is difficult to confirm whether the effects of reducing LAT1 expression on proliferation is indeed mediated by the inhibition of mTORC1. However, these effects are unlikely due to the potential positive effects of leucine on insulin secretion as these experiments were carried out in nutrient rich media containing a high concentration of glucose.

Discussion

In this report we provide evidence that System-L transporters, and in particular LAT1, play a critical role in β -cell signalling to mTORC1, the maintenance of β -cell function and proliferation. LAT1 is highly expressed in rat, mouse and human islets of Langerhans ((Figure 5) and (Fukushima *et al.* 2010; Nakada *et al.* 2014; Zhou *et al.* 2014)) and is also highly expressed in testicular sertoli cells, ovarian follicular cells and proximal to proliferative zones in the gastrointestinal mucosa (Nakada *et al.* 2014). In addition, LAT1 is widely expressed in non-epithelial cells showing its highest expression in endothelial cells forming the blood-brain barrier in the brain indicating that it is also important in transporting amino acids across endothelial/epithelial secretory barriers such as blood-

brain barrier (Mastroberardino *et al.* 1998; Verrey 2003). As LAT1 expression is upregulated in a range of tumours and is widely expressed in foetal tissue, LAT1 is thought to be particularly important in transporting amino acids into highly proliferating cells (Nakada *et al.* 2014; Yanagisawa *et al.* 2014). As LAT1 is an obligate amino acid exchanger, its activity is not only governed by extracellular amino acid concentration but also by the intracellular amino acid concentration controlled by, for example, metabolism and the activity and expression of other amino acid transporters (Meier *et al.* 2002; Verrey 2003). These include the sodium dependent system A transporters, such as SNAT2, which transport small neutral amino acids such as glutamine and alanine which act as substrates for LAT1 to drive the transport of BCAA and aromatic amino acid into the cell (Evans *et al.* 2007). The reported K_m of LAT1 for BCAAs is approximately 50-100 μ M whereas for leucine it is between 18-62 μ M (Meier *et al.* 2002). We show that the EC50 for leucine activation of mTORC1 in INS1E cells was approximately 200 μ M, well within the physiological relevant range of plasma leucine concentration, which is between 100-400 μ M. The difference between the EC50 for leucine-stimulated mTORC1 activation and the K_m for LAT1 could be due to factors independent of transport required for the activation of mTORC1 or they could be simply due to differences in cell systems and experimental conditions. Another possibility is that other amino acid transporters also play an important role in leucine uptake into β -cells, such as LAT4. LAT4 was also found to be highly expressed in islets (Figure 5) and this transporter has a K_m of approximately 3mM for leucine (Bodoy *et al.* 2005).

As the combined fasting concentration of System-L AA substrates is approximately 650 μ M (Bergstrom *et al.*; Kidney Int. 1990), LAT1 is likely operating near saturation. Thus changes in the expression of LAT1 will increase BCAA uptake. LAT1 expression is upregulated by growth factors via activation of mTORC1 but also by ER stress, probably via ATF4 (Harding *et al.* 2003; Liu *et al.* 2004; Wang *et al.* 2011a; Krokowski *et al.* 2013; Cnop *et al.* 2014). As β -cells undergo ER stress in the face of obesity and insulin resistance, it is possible that LAT1 expression is increased and that this in turn leads to increased mTORC1 activation and a compensatory increase in β -cell function and mass. Therefore, it is important to establish whether there are changes in LAT1 expression in β -cells during both the progression of obesity and type-2 diabetes.

Amino acid transporters have also been proposed to act as receptors that sense changes in the extracellular/intracellular amino acids concentration, possibly by monitoring amino acid flux through the transporter, to transduce an intracellular signal (Hundal & Taylor 2009). For example, SNAT2 has been proposed to act as a ‘transceptor’ and regulate PKB activity (Evans *et al.* 2007, 2008). Interestingly, BCH inhibited PKB activation. In addition, knock down of LAT1 also inhibited PKB activity. How System-L transporters influence PKB activity is unknown although it may be independent of the intracellular concentration of BCAA. Interestingly, the effects of BCH on signalling to mTORC1 and PKB are much stronger than that of amino acid withdrawal, despite the fact that amino acid withdrawal results in a greater effect on the intracellular level of leucine/BCAA. The reason for this is not entirely clear but a possible explanation is that signalling to mTORC1 and PKB is more responsive to the L-Leucine/transporter complex than it is to the free cytosolic L-Leu concentration (Hundal & Taylor 2009).

As BCAA can activate mTORC1, a kinase that stimulates increased pancreatic β -cell mass and function (for review see (Xie & Herbert 2012)), increased LAT1 expression or activity may be anti-diabetogenic. However, the chronic hyperactivation of mTORC1 inhibits insulin/growth factor signalling mediated by ribosomal protein S6 kinase (S6K) inhibitory phosphorylation of insulin receptor substrate (Um *et al.* 2004, 2006). Indeed, although β -cell specific mTORC1 gain-of-function mutant adult mice initially have increased β -cell function and mass (Shigeyama *et al.* 2008; Hamada *et al.* 2009) with age there is a decline in β -cell function and mass mediated by feedback inhibition of mTORC1 and the induction of autophagy (Shigeyama *et al.* 2008; Bartolomé *et al.* 2014). Interestingly, obese diabetic humans have increased circulating levels of BCAA and this is predictive of T2DM progression (Newgard *et al.* 2009; Fiehn *et al.* 2010; Wang *et al.* 2011b). Thus it is plausible that elevated BCAA may cause chronic activation of mTORC1 in β -cells. This could result in a critical switch from mTORC1 stimulating β -cell growth to causing β -cell dysfunction, death and the onset of diabetes. Moreover, it has recently been reported that increased amino acid flux mediated by increased AA transporter expression exacerbates ER stress induced β -cell death and diabetes in the Akita mouse (Krokowski *et al.* 2013). Hence although BCAA likely plays an important positive role in maintaining glucose homeostasis, chronic increase in circulating BCAA may be a critical

determinant in the development of type-2 diabetes. Although this report focuses on the role of LAT1 in β -cell signalling and function, LAT2 and LAT4 were also found to be expressed in both clonal β -cells and islets of Langerhans and thus are also likely to play important roles in the β -cell.

Author Contribution

TPH conceived and designed the study. AB helped in the design of the study and provided invaluable intellectual and technical advice. QC, VDB, JB, SC and TPH conducted the experiments. QC, VDB, and TPH analysed the results. TPH wrote the manuscript and all authors approved the final version of the manuscript.

Acknowledgements

We would like to thank Dr E Gomez for critical reading of this manuscript.

Figure legends

Figure 1. System-L amino acid transporters are required for signaling to mTORC1 in INS1E cells. **a** Serum starved INS1E cells were incubated for 30min in EBSS (20mM glucose, 2mM L-glutamine) supplemented with either 1x MEM essential amino acids (EAA) (+ System L-AAAs) or 1x MEM EAA minus the System-L amino acid substrates (- System L-AAAs) in the presence or absence of 100nM insulin +/- 2.5mM BCH as indicated. Proteins were resolved by SDS-PAGE, and Western-blotted using antisera against the proteins indicated. The results are graphically represented below showing the mean + S.E.M for three independent experiments. ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$; compared with insulin plus System-L amino acids. (A.U. = arbitrary unit). **b**) Serum starved INS1E cells were incubated for 30min in EBSS (20mM glucose, 2mM L-glutamine) supplemented with either 1x MEM essential amino acids (EAA) and 100nM insulin. The effect of the addition of BCH (+BCH) or the withdrawal of System-L amino acids ((-)L-AAAs) on intracellular amino acid concentration was determined by HPLC analysis. The results are mean + S.E.M for five independent

experiments. *, $P < 0.05$; and ***, $P < 0.001$ compared with control. **c)** L-[^3H]-Leucine uptake (in the presence of 0.8mM leucine) in the absence (- BCH) or presence (+ BCH) of 2.5mM BCH. The results presented are the mean + S.E.M of 3 independent experiments. ***, $P < 0.001$ compared with control.

Figure 2. The system-L amino acid substrate leucine is required for signaling to mTORC1 in INS1E cells. **a)** Serum starved INS1E cells were incubated for 30min in EBSS containing 20 mM glucose, 2mM L-glutamine supplemented with either 1 x MEM EAA (+ System-L AAs) or 1x MEM EAA minus System-L amino acids (- System-L AAs) in the presence or absence of each individual system-L amino acid substrate (single letter amino acid code) as specified. Where indicated, 100nM Insulin (insulin) was also included. **b)** BCH inhibits leucine-stimulated mTORC1 activation in INS1E cells. INS1E cells were pre-incubated in DMEM minus L-Leucine for 1 h. The cells were then incubated for a further 30 min in DMEM minus leucine supplemented with either 0.8mM or 0.4mM leucine in the presence or absence of 2.5mM BCH. **c)** Leucine stimulates mTORC1 in a dose-dependent manner. INS1E cells were pre-incubated in DMEM minus L-leucine for 1 h, followed by 30 min treatment in DMEM minus leucine supplemented with increasing concentration of L-leucine as indicated. Leucine concentration response curves and corresponding EC₅₀ are shown below. The results presented are the mean (+/- S.E.M) of at least three independent experiments. **d) INS1E cells were pre-incubated in DMEM minus L-Leucine for 1 h. The cells were then incubated for a further 30 min in DMEM minus leucine supplemented with either 0.8mM leucine in the presence or absence of 10 μM nifedipine.** In all cases, proteins were resolved by SDS-PAGE, and Western-blotted using antisera against the proteins indicated. All results are representative of at least three independent experiments.

Figure 3. The temporal kinetics of leucine-dependent mTORC1 activation. INS1E cells were pre-incubated in **a)** DMEM containing 0.8mM L-Leucine or **b)** DMEM minus leucine for 1 h followed by the incubation in **(a)** DMEM minus leucine or **(b)** DMEM containing 0.8mM L-Leucine for up to 30 min. **a and b)** Proteins were resolved by SDS-PAGE and Western-blotted using antisera against the proteins indicated. The results are the mean + S.E.M for at least three independent

experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, compared with control (i.e. 0 min time point). **c)** Left 2 bars: INS1E cells pre-incubated in DMEM+0.8mM Leu for 1 hr followed by incubation in DMEM minus Leu for 10min; right 2 bars: pre-incubated in DMEM minus Leu for 1hr followed by incubation in DMEM+0.8mM Leu for 10min. The cells were harvested and the intracellular concentration of leucine determined by HPLC.

Figure 4. System-L transporters play an important role in regulating islet signaling and function. Rat islets of Langerhans were incubated in DMEM minus L-Leucine for 1h prior to a 30 min incubation in DMEM plus or minus 0.8mM Leucine in the presence or absence of 2.5mM BCH. **a)** Proteins were resolved by SDS-PAGE, and Western-blotted using antisera against the proteins indicated. Graphical representation of the results are shown below. **b)** Insulin Secretion Assay performed by ELISA on the test medium collected at the end of above treatment. **c)** Islets of Langerhans were incubated in RPMI supplemented with 0.2% FBS for 24h. The medium was replaced with DMEM minus leucine or DMEM containing 0.8mM leucine in the presence or absence of 2.5mM BCH for 48 h. 2 μ Ci [3 H]-thymidine was added for the last 24h. **d)** Rat islets of Langerhans were incubated in DMEM minus L-leucine for 1h prior to a 30 min incubation in DMEM plus or minus 0.8mM leucine in the presence or absence of 2.5mM BCH. Cells were harvested for HPLC analysis and the intracellular leucine concentration determined as described in methods. **e)** L-[4,5- 3 H]-Leucine uptake in the presence of 0.8mM leucine in either the presence (+BCH) or absence (-BCH) of 2.5mM BCH. All results presented are the mean + S.E.M for at least three independent experiments. ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$.

Figure 5. LAT1 is highly expressed in primary islets. **a)** qPCR analysis of LAT1, LAT2, LAT3 and LAT4 expression in isolated rat islets of Langerhans expressed as fold change in expression of the target gene (LAT1, LAT2, LAT3 and LAT4) relative to the reference gene (18S RNA). The mean normalized expression (MNE) of the target genes was calculated using $2^{-\Delta\Delta CT}$ method. Expression values were multiplied by 10^6 for clarity of presentation. The results presented are the mean + S.E.M of three independent experiments. **b)** Rat islets of Langerhans were transfected with siRNAs against:

CD98(SLC3A2) or scrambled siRNA as control. After 72 h the cells were incubated in DMEM minus Leucine for 1 h, followed by the re-addition of 0.8mM Leucine for 30 min. Proteins were resolved by SDS-PAGE, and Western-blotted using antisera against the proteins indicated. The results are graphically represented below showing the mean + S.E.M for three independent experiments. ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$ (A.U. = arbitrary unit).

Figure 6. LAT1 couples to mTORC1 signalling in islet cells. Rat islets of Langerhans were transfected with siRNAs against: a) LAT1 (Slc7a5) (INVITROGEN s132356) (siLAT1-A) or scrambled siRNA (siScr) as control; and b) LAT1 (Slc7a5) (Dharmacon, SMART POOL on TARGET) (siLAT1-B) or scrambled siRNA (siScr) as control. After 72 h the cells were incubated in DMEM minus Leucine for 1 h, followed by the re-addition of 0.8mM Leucine for 30 min. Proteins were resolved by SDS-PAGE and Western-blotted using antisera against the proteins indicated. The results are graphically represented below showing the mean + S.E.M for three independent experiments. ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$ (A.U. = arbitrary unit). **c and d)** Quantification of LAT1 expression in dispersed rat islets by qPCR after 72 h of transfection with (c) LAT1 (Slc7a5) (Dharmacon, SMART POOL on TARGET) or (d) (INVITROGEN s132356). Mean + S.E.M for three independent experiments. ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$ (A.U. = arbitrary unit).

Figure 7. LAT1 is required for islet cell function and proliferation. Dispersed rat islets of Langerhans were transfected with siRNAs against: a)) LAT1 (Slc7a5) or scrambled siRNA as control. After 72 h the cells were incubated in DMEM minus Leucine for 1 h followed by the re-addition of 0.8mM Leucine for 30 min. An Insulin ELISA assay from on media collected after treatments. b) Dispersed rat islets of Langerhans were transfected with LAT1 (Slc7a5) or scrambled siRNA as control and incubated for 72h. $1\mu\text{Ci/ml}$ ^3H -thymidine was added in the last 24 h.

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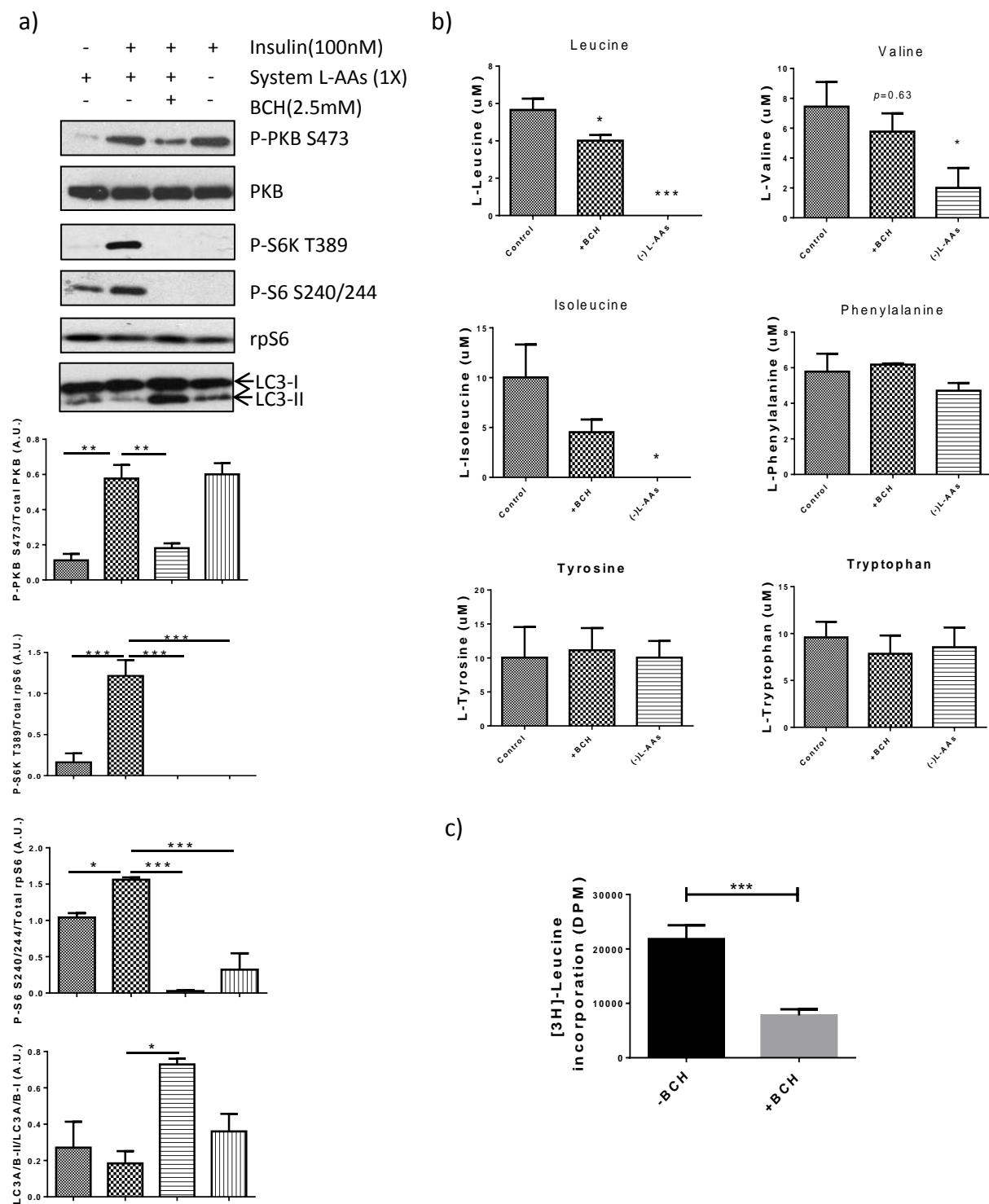


Figure 1

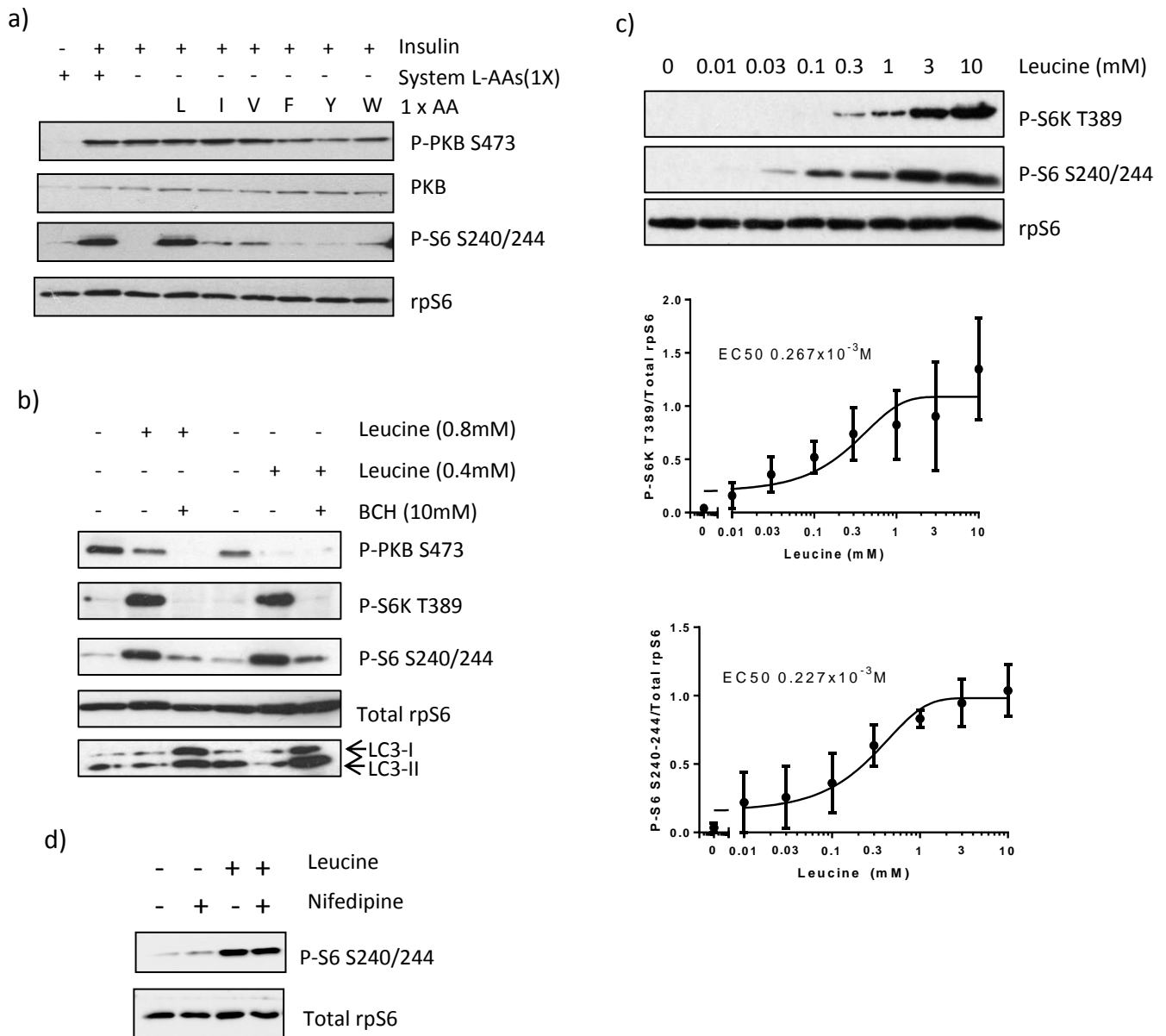


Figure 2

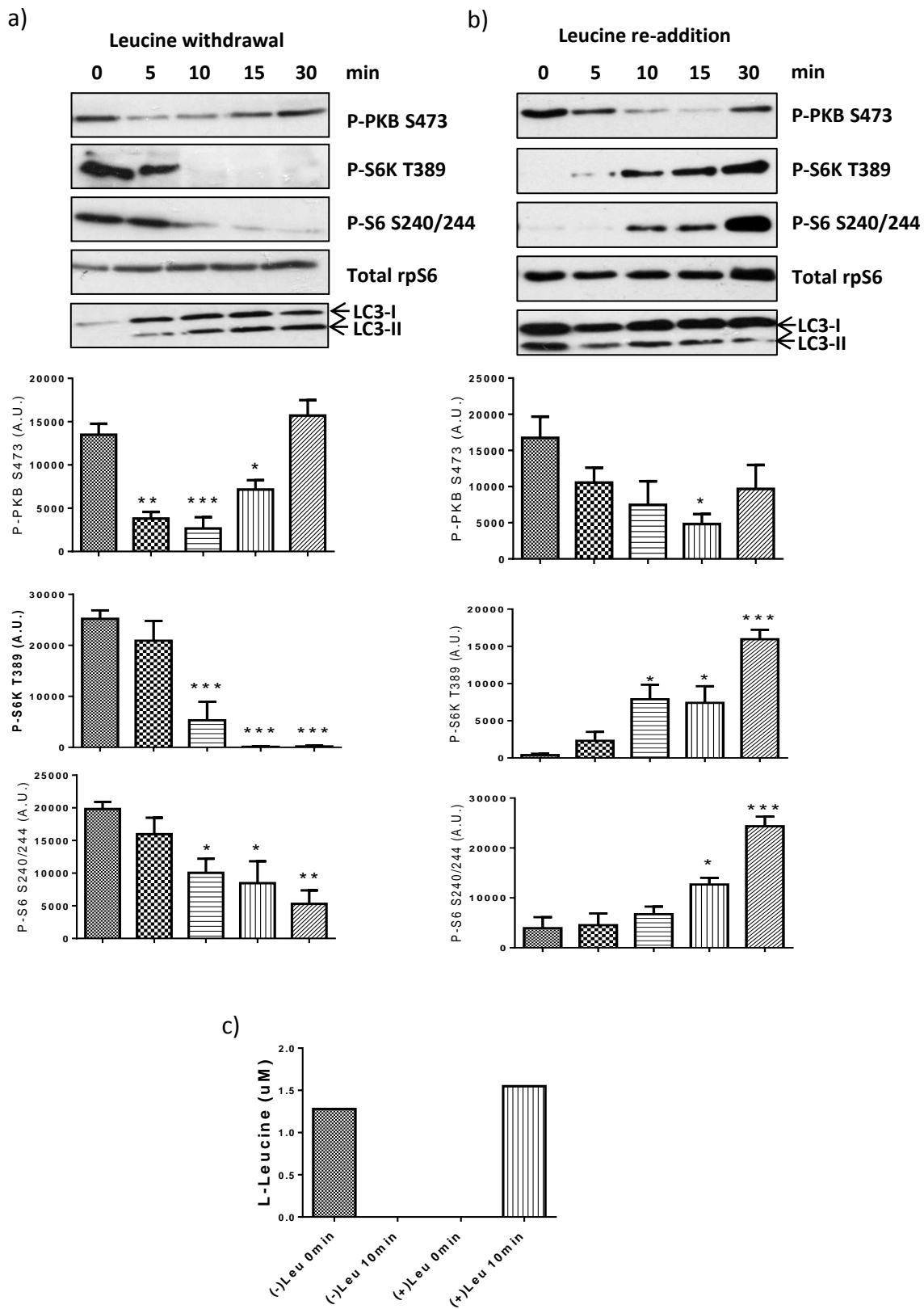


Figure 3

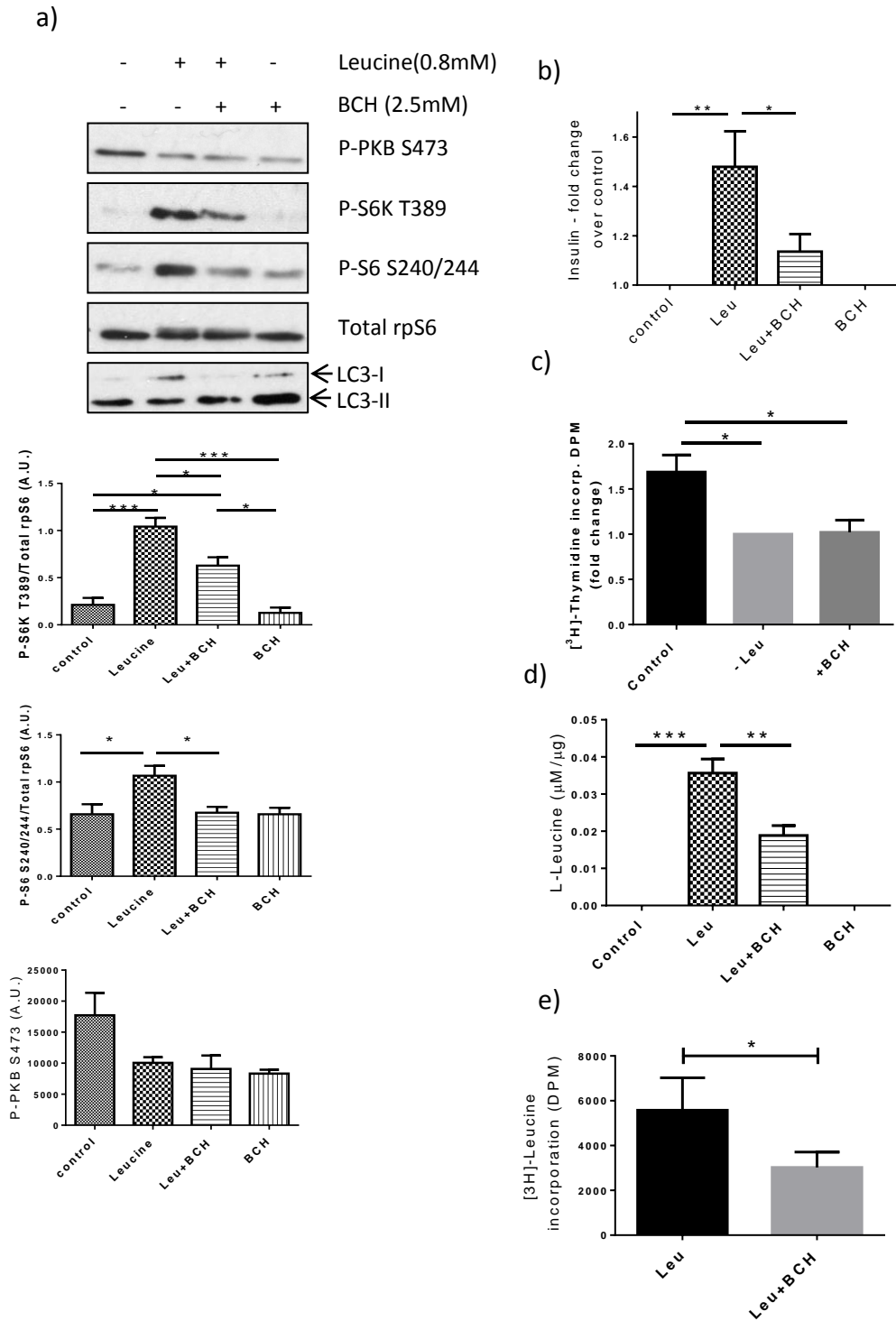
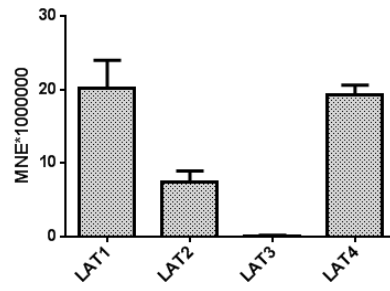


Figure 4

a.



b.

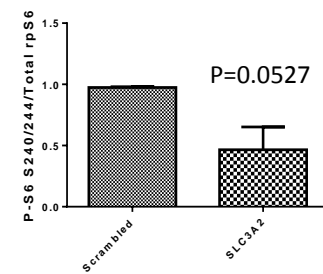
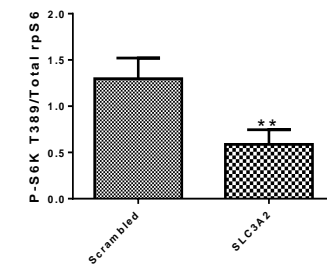
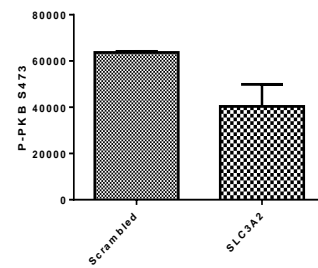
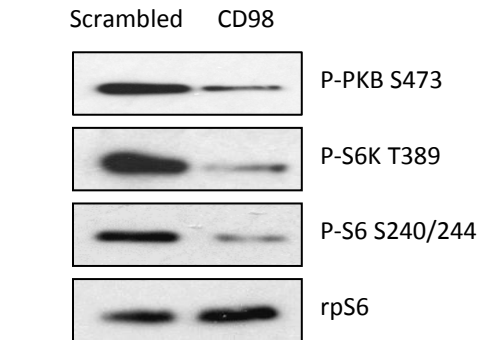


Figure 5

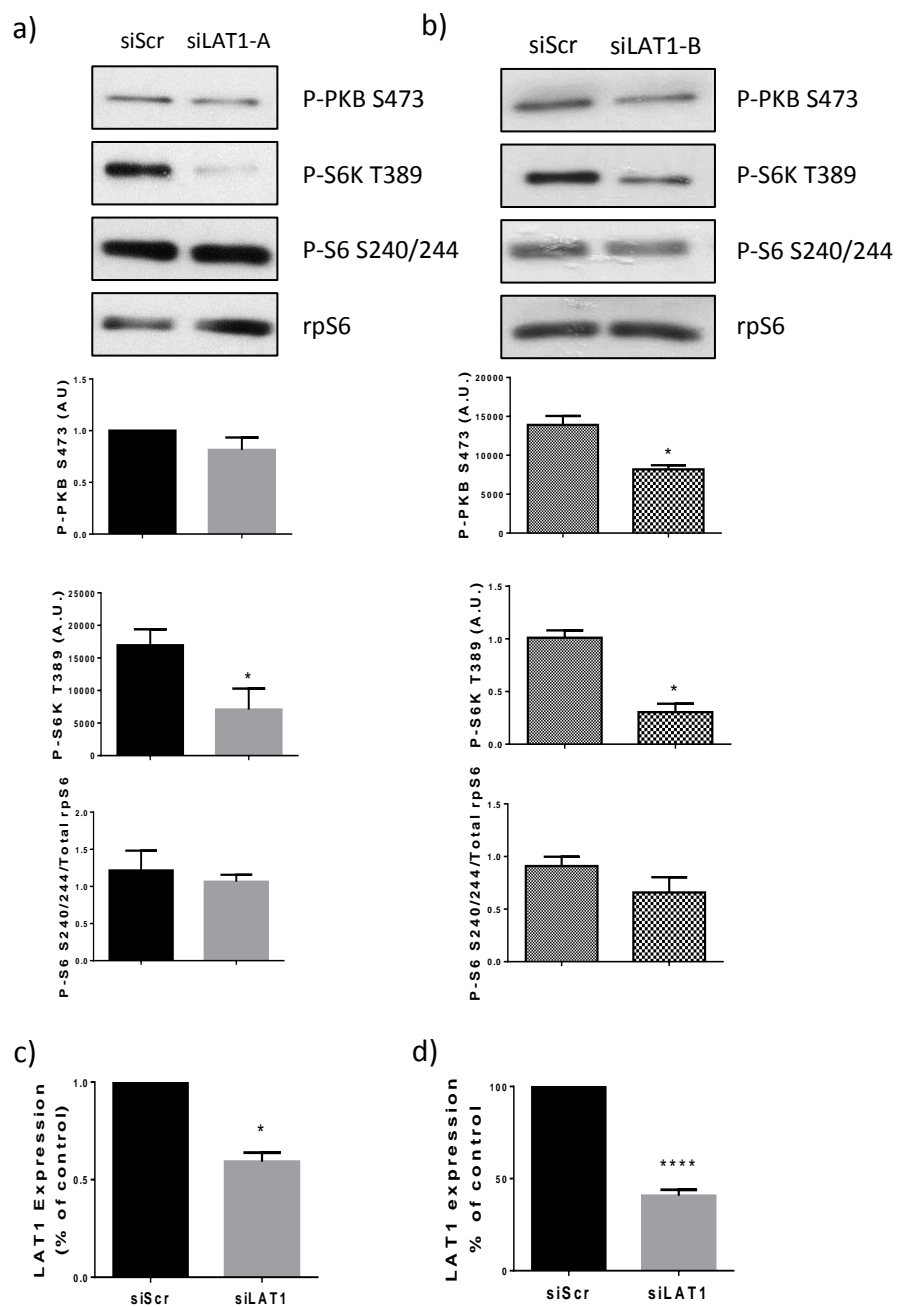
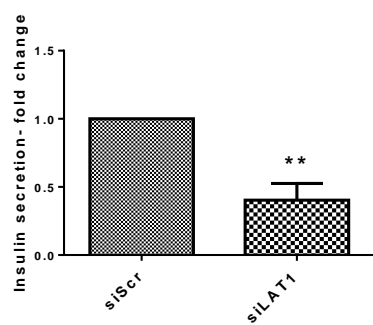


Figure 6

a)



b)

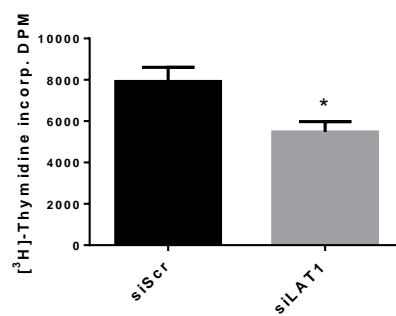


Figure 7